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Relationship between production of carrot somatic embryos and dissolved oxygen concentration in liquid culture

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Abstract

To evaluate the relationship between somatic embryogenesis and dissolved oxygen concentration, somatic embryo cultures of carrot (*Daucus carota* L.) were cultured under various dissolved oxygen concentration levels (bubble free aeration with 4%, 7%, 20%, 30%, and 40% oxygen in flasks). The system used allows dissolved oxygen concentration control without bubble aeration or mixing speed modification. The total number of somatic embryos was not affected by the dissolved oxygen (DO) concentration tested. Even if globular-stage embryos were induced at a low level of oxygen aeration, heart-stage embryo formation was still repressed. Oxygen enrichment (20%, 30% and 40% oxygen) enhanced torpedo and cotyledonary-stage embryo production. The oxygen-enriched aeration was effective in promoting the growth of the late developmental stages. Sugar consumption did not increase when the oxygen concentration was enriched above the ambient level. The number of heart-stage embryos increased as oxygen concentration increased up to the 7% level, while above the 20% level no change in production was observed. The production of cotyledonary-stage embryos was directly related to oxygen concentration. These results support that oxygen-enriched aeration provides oxygen to the low oxygen areas in somatic embryo. After the heat-stage embryos, which were grown at the 7% level were transferred to a flask with ambient, they developed an elongated root part and eventually grew to normal plantlets.

Abbreviations: DO – dissolved oxygen; 2,4-D – 2,4-dichlorophenoxyacetic acid

Introduction

Labor-reducing automation and techniques for scaling up plant micropropagation are essential for future strategies of transplant production. Somatic embryogenesis is a technique used to produce large numbers of individual embryos, and has received much interest as a means to produce material for artificial seeds. The commercialization of artificial seeds or transplants using somatic embryos requires the development of a bioreactors system for large-scale production. Bioreactors enable the measurement and control of culture conditions (DO, pH, temperature, electric conductivity, oxidation-redox potential, mixing rate, etc.) in liquid medium.

There have been several reports on somatic embryo production systems using a bioreactor (Preil et al., 1988; Cazzulino et al., 1991; Gupta et al., 1991; Denchev et al., 1992; Ducos et al., 1993; Molle et al., 1993; Harrell et al., 1994; Ibaraki et al., 1995). However, the effect of physical environmental factors such as pH (Jay et al., 1994), shear stress (Archambault et al., 1994), osmotic pressure, mass transfer rate, and DO concentration on somatic embryo culture has not been investigated in detail. DO concentration is one of the most important environmental factors when growing plants in liquid systems, since oxygen is only slightly soluble in water (8.0 mg l^{-1} at 25 °C, 1 atm, and 21% oxygen in ambient air). The relationship between DO concentration and somatic

embryogenesis is not clear from the results reported in previous studies. Kessell and Carr (1972) reported that a DO concentration below a critical level of 16% DO was essential for the production of carrot somatic embryos in a 4 l bioreactor mechanically stirred at 90 rpm. Carman (1988) concluded that a low oxygen gas level increased the number of wheat embryos. Stuart et al. (1987) induced alfalfa somatic embryos using a 2 l bioreactor, stirred at 100 rpm by an impeller and aerated by a sparger at 1.8 l min^{-1}. They indicated that somatic embryos of alfalfa can be regenerated at high DO concentrations, at least 70% DO, in a bioreactor. Jay et al. (1992) reported that the production of carrot somatic embryos in a 3 l mechanically stirred bioreactor operated at 50–150 rpm depended on the biomass concentration. Two cultures were produced at constant DO concentrations of 10% and 100% DO, using a controlled gas mixing system and a constant bubble aeration rate of 0.09 vvm (volume air per volume culture per minute). After 20 days, the yields were 170 embryos ml^{-1} and 600 embryos ml^{-1} in the 10% and 100% DO cultures respectively. Archambault et al. (1994) studied embryogenic cultures of a transformed *Eschscholtzia californica* cell line in an 11 l helical ribbon impeller bioreactor. They showed that the best production of somatic embryos was achieved by operating the bioreactor at 60 rpm maintaining the DO concentration at 20% DO, using surface oxygenation only (0.05 vvm, 1.4 h^{-1} k_La). The high DO concentrations of 60% DO favored undifferentiated biomass production at the expense of the slower growing somatic embryos. Okamoto et al. (1996) reported the effect of the oxygen concentration in the aeration gas on plantlet regeneration from rice cells in bioreactor cultures. They showed that the efficiency of regeneration in cultures aerated with gas mixtures of over 40% oxygen was higher than that in a flask culture. Shigeta et al. (1996) carried out somatic embryo production using a 250-ml Spinner flask and a 2 l jar-fermentation system. They reported that a concentration of at least 80% DO was needed for the development of globular and heart-stage embryos during the first week of culture. However, subsequent development into torpedo-stage embryos proceeded even at low DO concentrations.

The objective of this study is to clarify the role of DO concentration during the somatic embryogenesis phase in the production of somatic embryos. The differences in the experimental results reported could be attributed to the culture system, medium formulation, plant species, and inoculated embryogenic cell (cell density, cell cluster size, washing of residual 2,4-D and cell line used). In particular, the types of DO concentration regulation methods used differed in the mixing speed and aeration rate during the culture period. Therefore, in the research reported, the results were also effected by the shear in the liquid medium as well as the DO concentration. To investigate only the relationship between DO concentration and somatic embryogenesis, we used a liquid culture system, which did not require either bubble aeration or changing the mixing rate to control the DO concentration.

Materials and methods

Plant material

In this experiment, carrot (*Daucus carota* L. cv. 'Kinko-yonsun') was used as a large-scale production model because it has one of the most established culture protocols. The cell suspensions were initiated from hypocotyl segments of the seedling, which was germinated in Gamborg B5 medium (Gamborg, 1970). The hypocotyl segments of a seedling were cut into 5 mm pieces and transferred into 10 ml Gamborg B5 liquid medium supplemented with 30 g l^{-1} sucrose and 0.5 μM 2,4-dichlorophenoxyacetic acid (2,4-D) in 50 ml Erlenmeyer flask at 26 °C in the dark. The pH of medium was adjusted to 5.5, before autoclaving at 120 °C for 20 min. After two months, induced calli were transferred into 100 ml of fresh medium which is same composition as the callus induction medium. Suspension was rotated in 300 ml Erlenmeyer flask at 80 rpm in the dark at 26 °C. A 10 ml cell suspension was then transferred to fresh Gamborg B5 medium containing 2,4-D and subcultured at 2-week intervals under the same conditions as mentioned above for 2 to 6 months. Somatic embryo induction was obtained according to the method described by Fujimura and Komamine (1979). The 14-day-old carrot cell suspension was sieved through a 63 μm steel screen and subsequently through a 32 μm screen. Cell clusters retained on the 32 μm screen were layer on a Ficoll (15%, w/v in water containing, 8 ml in total volume) in a 10 ml tube and then centrifuged at 100 *g* for 5 min. 2% sucrose was added to the Ficoll solution to adjust the osmotic pressure. Subsequently embryogenic cell clusters were washed 7 times in fresh Gamborg B5 medium lacking 2,4-D by centrifugation at 100 *g* for 5 min. Embryogenic cell clusters were transferred to

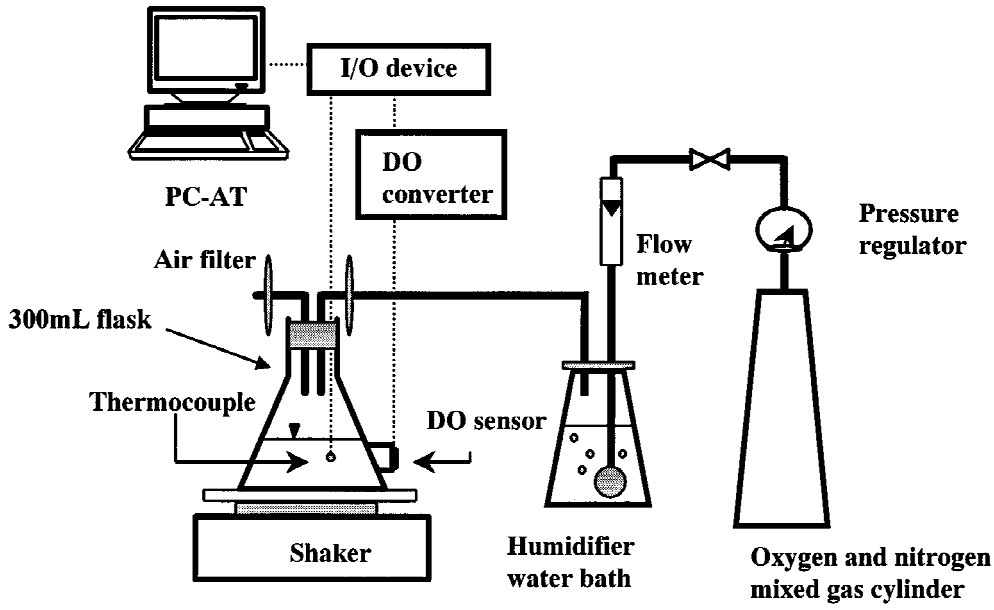

Figure 1. Schematic diagram of the system.

a same medium (100 ml) and grown in 300 ml Erlenmeyer flasks. The initial density of the cell cluster suspensions was 250 cell clusters ml^{-1}.

The culture system

Figure 1 is a schematic drawing of the system in which the effect of DO concentration on carrot somatic embryogenesis was studied. In rotary flask-culture, mild stirring occurs, but the cell or tissues are not damaged (Nishimura et al., 1993). A rotary flask-culture system was used which achieves the desired DO concentration without the mixing speed and aeration rate being varied. DO concentration was regulated by altering the oxygen gas concentration in the headspace of these flasks. Therefore, the effect of DO concentration can be isolated and measured, while shear stress is constant. The system was equipped with 20 flasks on a shaker, and each treatment was replicated four times. One flask of each treatment was equipped with a galvanic type DO probe, (SOM, ABLE Co., Japan), DO probe port, and a thermocouple to measure medium temperature for the calibration of DO. The mixture of oxygen and nitrogen gas was humidified by bubbling through deionized water, and supplied into each flasks fitted with an air filter. During the culture period, the aeration rate into the flasks was maintained constant at 10 ml min^{-1}. The oxygen mass transfer rate (k_La) of the flasks was measured using the nitrogen gassing out method (Van't Riet, 1979) and was 7.5 h^{-1}. In these experiments, k_La was higher than that of the 4.3–6.7 h^{-1} used by Okamoto et al. (1996) (500 ml and 2 l bioreactor). Five oxygen gas concentrations levels (4%, 7%, 20%, 30% and 40%, the remainder nitrogen) aeration were tested to evaluate the effect of DO concentration level on somatic embryogenesis. The experiment duration was 23 days. At the end of the experiment, the DO concentration levels of 4% and 7% oxygen were changed to ambient level, and somatic embryo culture was performed to investigate the effect of DO concentration change during somatic embryogenesis phase.

Quantification of somatic embryo

Somatic embryos progress through four stages of development: globular stage, heart stage, torpedo stage, and cotyledonary stage, while they are undergoing tissue differentiation. The stages are based on the overall embryo shape. In general, carrot somatic embryos remain in the torpedo stage for a few days before progressing to the cotyledonary stage. Cotyledonary-stage embryos have an elongated radicle and hypocotyl, which tends to exhibit hyperhydricity in liquid medium. Moreover, the elongated cotyledonary-stage embryos intertwine with each other in the bioreactor and form clumps. If somatic embryos are to be used as material for artificial seed, it is therefore desir-

able to harvest somatic embryos at the torpedo stage. 1 ml samples were collected from the all suspensions every two or three days and the somatic embryos were counted using a microscope, and classified into the various developmental stages. When cotyledonary-stage embryos began to appear, the somatic embryo suspensions were sieved through an 850 μm screen, and the number of cotyledonary-stage embryos was counted at the same time each day. The smaller embryos remaining in the suspensions were allowed to continue to grow.

Assay of the culture medium

On the last day of the culture period, culture medium was sampled, and sucrose, fructose and glucose were assayed using a high-performance liquid chromatograph. The medium was injected into a packed column (SGSO-252, 8 mm i.d. 250 mm, Yokogawa-hokushin Co., Japan) and monitored with a differential refractometer (ERC-7512, Erma Co., Japan).

Results

Changes of the medium DO concentration in the flasks under various aeration conditions

Figure 2 shows the changes in the medium of DO concentration under various aeration conditions. The DO levels during the somatic embryo culture dropped somewhat from the initial saturated DO level in each treatment. However, these drops were so low that the different DO levels remained unchanged the culture period. Average DO concentrations of each treatment were 2.0 mg l^{-1}, 2.8 mg l^{-1}, 7.1 mg l^{-1}, 10.8 mg l^{-1} and 14.0 mg l^{-1} for 4%, 7%, 20%, 30% and 40% oxygen gas respectively. For the 40% oxygen treatment, DO level increased later in the culture period. This was attributed to the reduced oxygen uptake after the harvest of cotyledonary-stage embryos.

Time course of the number of somatic embryos

Figure 3 shows the effect of DO concentration on carrot somatic embryogenesis in terms of the number of somatic embryos at each stage (globular heart, torpedo, and cotyledonary). The rate of increase in the total number of somatic embryos (all developmental stages) was not significantly affected by the DO concentration, as shown by the similarity of the curves for all treatments (Figure 3A). The total number of somatic embryos reached a constant level after day 17 for all treatments, with the exception of the 4% oxygen treatment. The day at which somatic embryos began to appear at all developmental stages also was not affected by the DO concentration (Figure 3B–E). In the 20%, 30%, and 40% oxygen treatments, the number of globular-stage embryos increased until day 15, and decreased as the somatic embryos progressed into the heart stage (Figure 3B). However, in the 4% and 7% oxygen treatments, the number of globular-stage embryos did not decrease during the culture period (Figure 3B), and the number of heart-stage embryos formed was lower than that in the higher oxygen treatments (Figure 3C). The number of torpedo-stage embryos increased clearly at high DO concentrations (20%, 30%, and 40% oxygen). Few torpedo-stage embryos were observed in the suspension aerated with 4% oxygen (Figure 3D). Figure 3E shows the cumulative number of cotyledonary-stage embryos harvested by staining through an 850 μm steel mesh. The oxygen-enriched aeration resulted in enhanced productivity of cotyledonary-stage embryos.

Somatic embryos cultured for 23 days in various DO concentrations are shown in Figure 4. In the 4% oxygen treatment, globular-stage embryos were observed, but formation of the cotyledonary part did not develop (Figure 4A). In the 7% oxygen treatment, cotyledonary part was observed, but the radicle and hypocotyl parts were not elongated (Figure 4B). Elongation of the somatic embryos was promoted by higher oxygen concentration (Figure 4C–E).

Three days after the 7% oxygen treatment was changed to ambient level, many embryos developed an elongated radicle part (Figure 5A) and eventually grew to normal plantlets (Figure 5B). In the case of the 4% oxygen treatment, root elongation was also observed after transfer to ambient level, although the embryos did not develop to the cotyledonary stage (Figure 5C).

Relationship between sugar consumption and oxygen concentration

Figure 6 shows sugar concentration in the media at the end of the 23-day culture period. In most publication dealing with the uptake of sucrose by cell cultures, it is assumed that the sucrose molecules supplied into the culture medium are hydrolyzed into glucose and fructose by a cell wall or plasmalemma located invertase (Desjardins et al., 1994). Although sucrose was completely hydrolyzed to glucose and fructose in the 40% oxygen treatment, glucose and

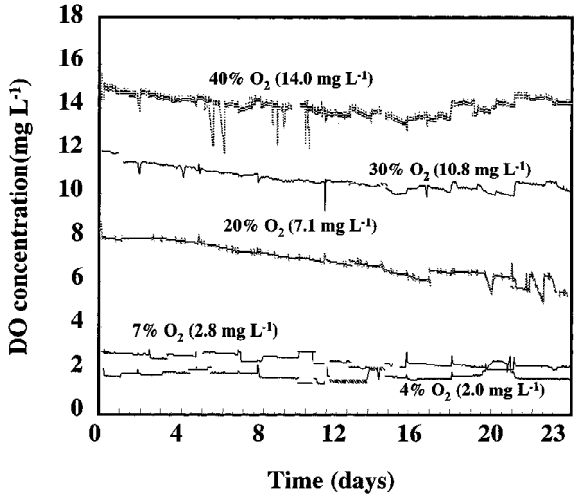

Figure 2. Changes of medium DO concentration in flasks under various aeration conditions.

fructose remained in the media. This result suggests that there was no carbon source exhaustion on this somatic embryo culture. Figure 7 shows the relationship between oxygen concentration and sugar consumption during the culture period. Somatic embryos preferentially consumed glucose relative to fructose for each treatment. Sugar consumption by the somatic embryos increased with rising oxygen concentration from 4% to 20%. However, at oxygen concentration above 20%, sugar consumption was unaffected by the raised oxygen level.

Relationship between somatic embryogenesis and oxygen concentration

Figure 8 shows the relationship between somatic embryogenesis and oxygen concentration. The number of heart-stage embryos increased sharply with increased oxygen concentration, when the range of lower oxygen concentration was raised up to 7%. However, above 20% oxygen, oxygen enrichment did not improve productivity of heart-stage embryos (Figure 8A). In contrast, the cumulative number of cotyledonary-stage embryos increased linearly with rising oxygen concentration (Figure 8B).

Discussion

Respiratory processes should be considered a relevant factor to affect DO level. Atwell and Greenway (1987) reported that many oxygenase activities including cytochrome oxidase were affected by reduced oxygen. The intrinsic K_m (half of the critical oxygen concentration) for cytochrome oxidase-mediated respiration is probably quite low, less than about 0.01 mg l^{-1} (Jackson and Drew, 1984). However, in our study the restriction of somatic embryo production occurred at higher DO concentration than 0.01 mg l^{-1}.

Furthermore, the effect of oxygen enrichment on the somatic embryo development was remarkable at later stages (torpedo and cotyledonary-stage embryos). Huang et al. (1992) suggested that the size of somatic embryos have increased and thus led to possible mass transfer limitations. Since oxygen is consumed by cell respiration during the transport through the somatic embryo, the oxygen concentration level within the somatic embryo may be lower than the bulk solution DO level. This oxygen concentration gradient depends on the size, geometry and respiration activity of somatic embryo. The size of the later developmental stage embryos was much larger than the globular-stage and heart-stage. Therefore, the DO concentration within somatic embryo might be below the critical level at later developmental stages if the bulk DO level is low, and the oxygen-enriched aeration promoted the production at the later stages. The experimental results shown in Figure 8 support this hypothesis. Similar results were reported for rice (Okamoto et al., 1996), and lily (Takahashi et al., 1992).

Our results are in contrast to those obtained by Kessel and Carr (1972) and Archambault et al. (1994) which showed that oxygen limitations promoted somatic embryogenesis. However, the somatic embryo

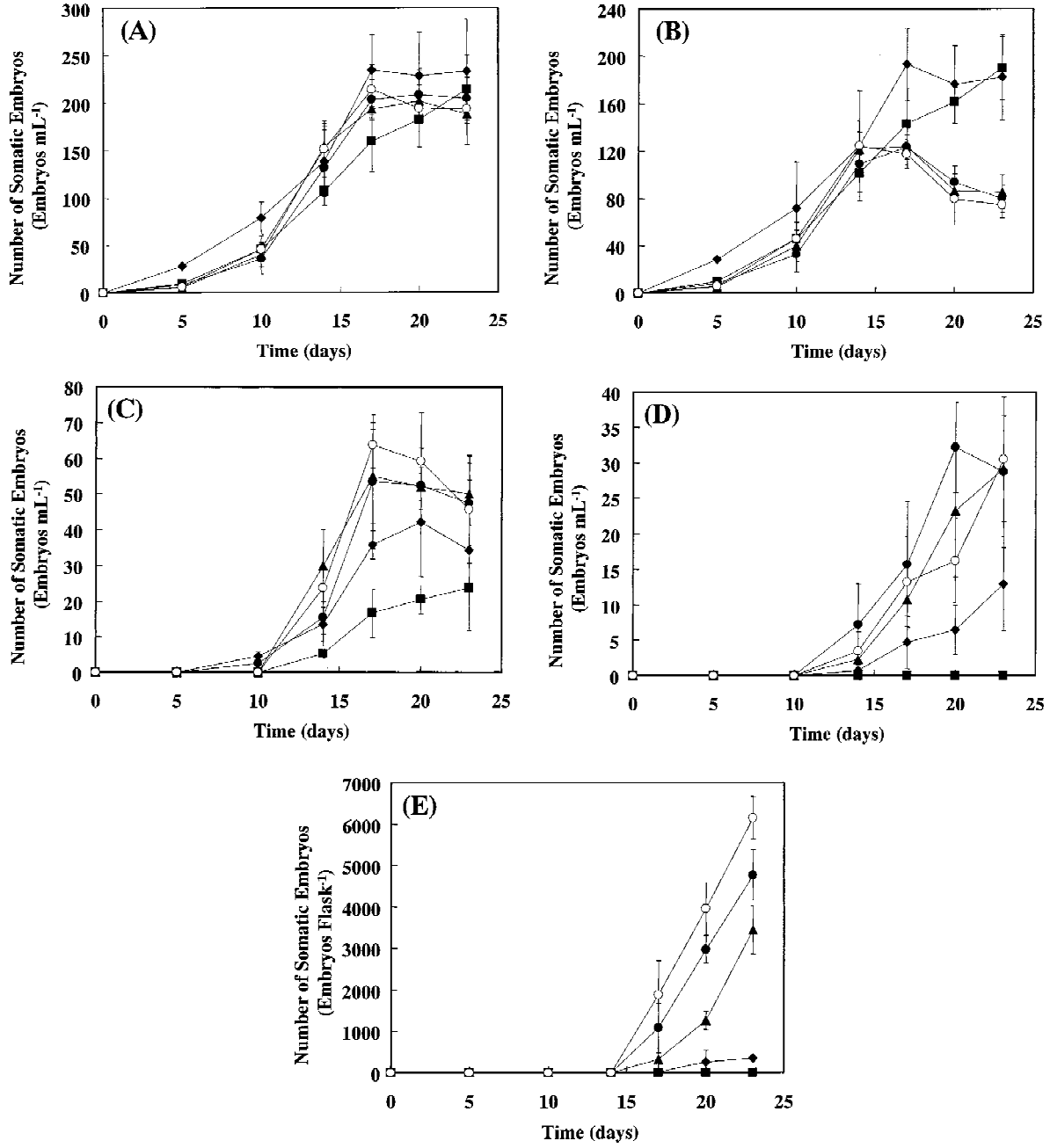

Figure 3. Time course of the mean number of somatic embryos per unit volume of the culture medium. (*A*) Total somatic embryos, (*B*) Globular-stage embryos, (*C*) Heart-stage embryos, (*D*) Torpedo-stage embryos, (*E*) Cotyledonary-stage embryos: Cumulative number of cotyledonary-stage embryos harvested by straining through a 850 μm steel mesh. Bar represent standard errors (n=4). Symbols: ■ 4% oxygen aeration, ♦ 7% oxygen aeration, ▲ 20% oxygen aeration, ● 30% oxygen aeration, ○ 40% oxygen aeration.

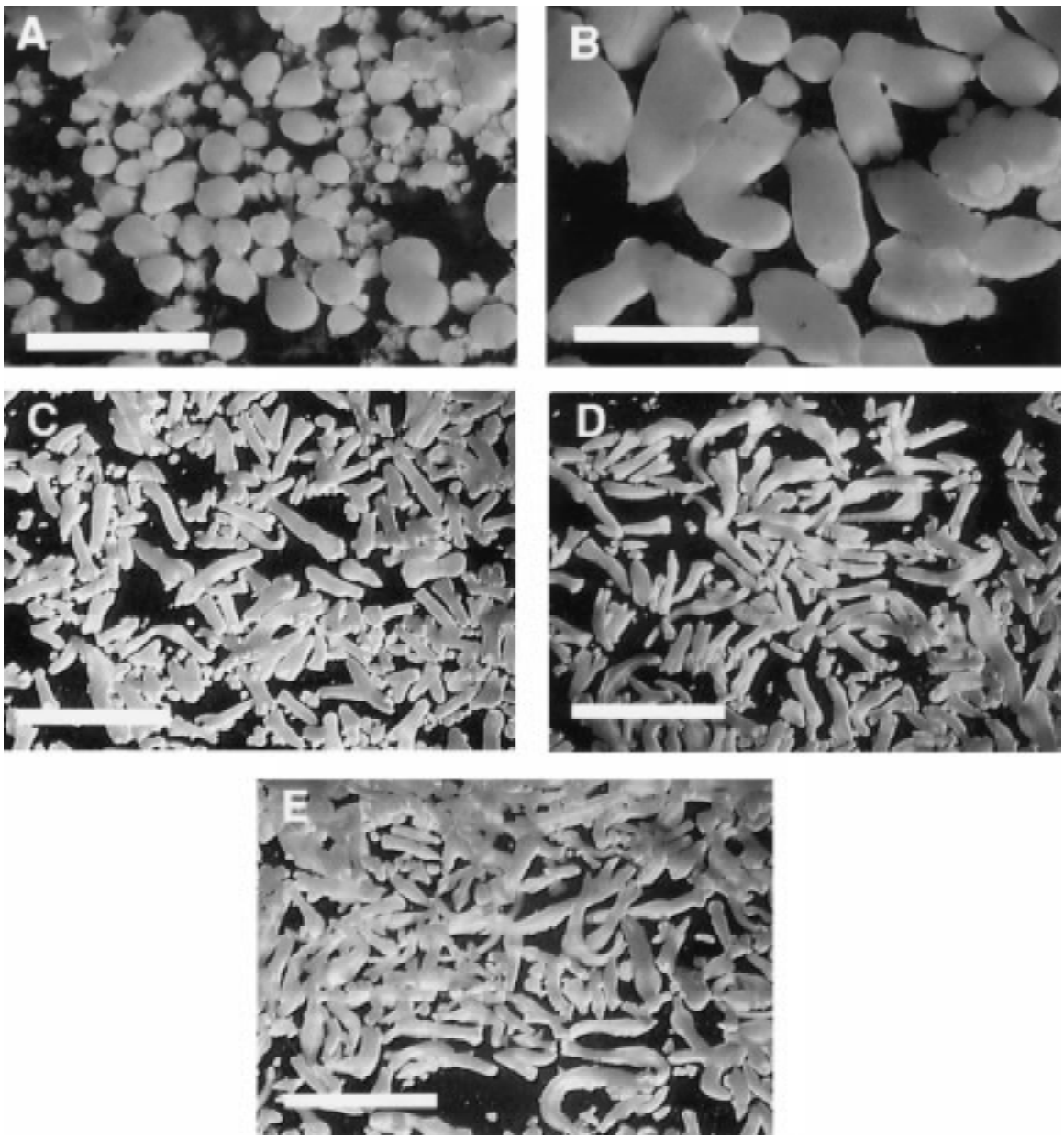

Figure 4. Somatic embryos harvested on day 23. (*A*) 4% oxygen aeration, (*B*) 7% oxygen aeration, (bar=1 mm), (*C*) 20% oxygen aeration, (*D*) 30% oxygen aeration, (*E*) 40% oxygen aeration, (bar=5 mm).

response to DO concentration may be dependent on plant species, cell density, cell cluster size, washing of residual 2,4-D and cell line used. The volume of the culture medium, the bioreactors design, and the airflow is also important parameters that will influence the oxygen transfer rate. These parameters differed among the previous studies, and could be considered as main causes of the contradictory results. The technique used to regulate oxygen concentration may have also lead to the differences in results. Kessel and Carr (1972) used a method of supplying oxygen by changing the sparging aeration and the agitation speed. Their results might include the effect of hydrodynamic stress. In our experiment, we were able to maintain the set point oxygen concentration with bubble free aeration, without the mixing speed modification or the

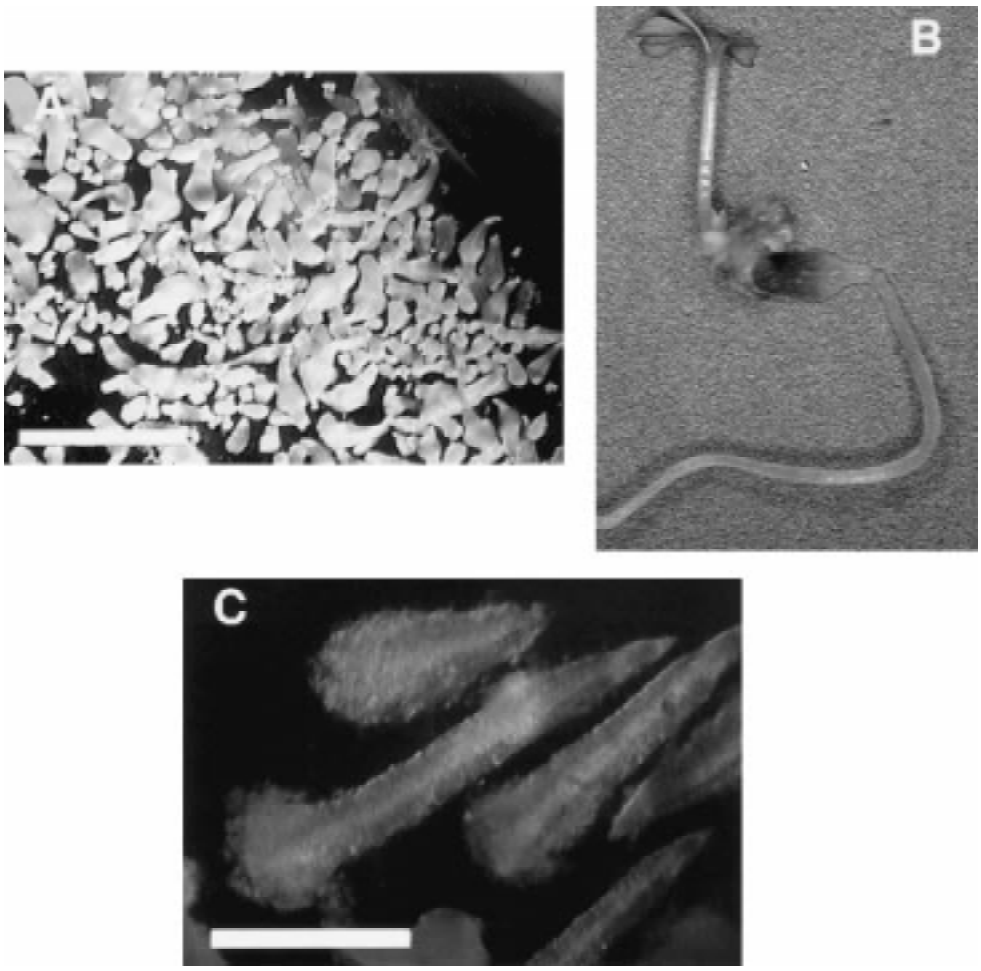

Figure 5. Somatic embryos aerated at ambient gas level after culturing in flasks which were aerated at 7% and 4% oxygen for 23 days. (*A*) Somatic embryos after culturing aerated 7% oxygen for 23 days. (bar=5 mm), (*B*) Regenerated plantlet from a somatic embryo from (*A*), (*C*) Somatic embryos at ambient gas level after culturing at 4% oxygen for 23 days. (bar=1 mm).

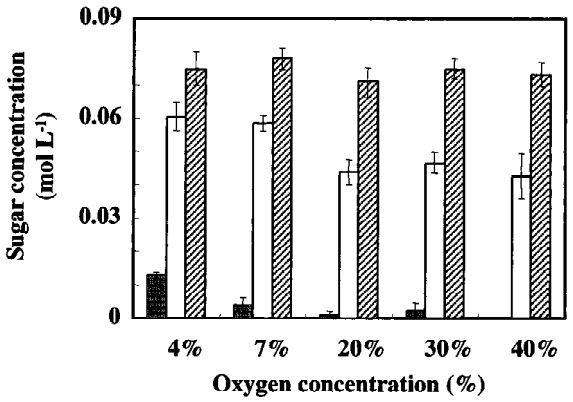

Figure 6. Sugar concentration in medium at end of the culture period (23 days). Symbols: U Sucrose, □ Glucose, R Fructose.

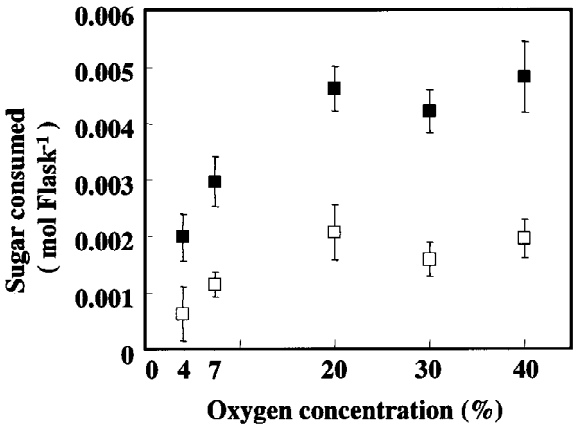

Figure 7. The relationship between oxygen concentration and sugar consumption during culture period (23 days). Symbols: ■ Glucose, □ Fructose.

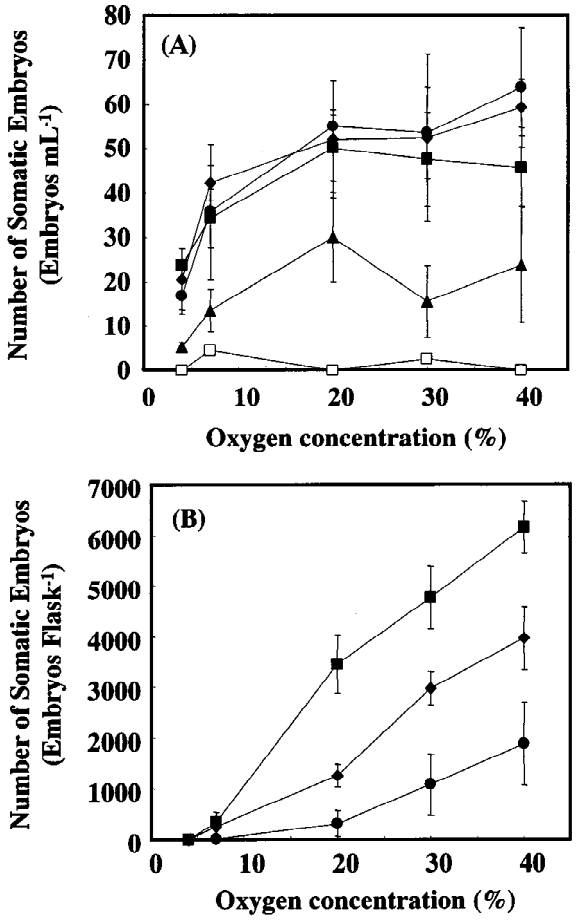

Figure 8. The relationship between oxygen concentration and somatic embryogenesis. (*A*) Heart-stage embryos, (*B*) Cumulative number of cotyledonary-stage embryos harvested by straining through a 850 μm steel mesh. Symbols: □ Day 10, ▲ Day 14, ● Day 17, ♦ Day 20, ■ Day 23.

sparging aeration. Thus, under our conditions, all differences in embryo development should be only due to the effect of varying the DO concentration.

With the 4% treatment, globular-stage embryos did not develop to cotyledonary-stage embryos even though they were transferred to a flask, and aerated with 20% oxygen. On the other hand, somatic embryos with 7% oxygen treatment developed into cotyledonary stage after they were transferred to a flask aerated with air, and subsequently, developed to plantlets on solid medium. This suggested that use of DO control dependent on developmental stage might provide an alternative for synchronization on somatic embryo production without filtration.

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